DH44-R2/CRFR as a Functional Target of miR-34 in Models of Alzheimer's Disease

Alzheimer's disease is the most common form of age-related neurodegeneration, accounting for as many as 70% of dementia patients (Brookmeyer et al. *Alzheimers Dement* 2011). It is characterized by the progressive deterioration and eventual death of neurons in the brain. This cellular death is associated with the accumulation of both intracellular and extracellular protein aggregates. Alzheimer's specifically accumulates aggregates of the amyloid-β protein thought to be toxic to neurons. Other inherited neurodegenerative disorders such as Huntington's and Parkinson's disease have similar aggregates that play key roles in the degeneration process and serve as histological markers of disease (Benilova et al. *Nat Neuro* 2012). Aggregated proteins often have long poly-glutamine (polyQ) tracts that are sensitive to mis-folding. The mechanisms that initiate pathological neurodegeneration are not well understood (Bilen et al. *Mol Cell* 2006). Therefore, gaining insights into these processes will promote the development of successful therapeutics to treat neurodegenerative disorders.

Recently, it has been shown that the down-regulation of a group of microRNAs (miRNA) normally expressed in the adult brain enhances neurodegeneration (Bilen et al. *Mol Cell* 2006). MicroRNAs are 21-23 nucleotide non-coding RNAs that bind to the 3'UTR of specific mRNA targets resulting either in target degradation or translational repression (Forman and Coller *Cell Cycle* 2010). The miRNA mir-34 has been identified in several model systems as a key player in neurodegeneration. In humans, down-regulation of mir-34 occurs at early stages of Parkinson's disease (Miñones-Moyano et al. *Hum Mol Gen* 2011). In *Drosophila melanogaster* it has been demonstrated that down-regulation of mir-34 results in neurodegenerative phenotypes; loss of mir-34 in fruit flies leads to increased neural tissue deterioration visualized by vacuoles or "holes" in the brain and formation of inclusion bodies containing the stress-induced chaperone Hsp-70. These phenotypes are exacerbated in backgrounds already sensitized to neurodegeneration (Liu et al. *Nature* 2012). One such background involves high expression of a transgene encoding a pathogenic protein called ataxin-3, which contains long polyQ tracts. This "polyQ" protein is extremely susceptible to aggregation and thus mimics several of the phenotypes seen in Alzheimer's and other protein-misfolding diseases (Warrick et al. *Cell* 1998).

Mir-34 has several putative targets in *Drosophila melanogaster*. Eip74A, an ecdysone-inducible ETS transcription factor, has previously been identified as a functional target of mir-34 in the progression of neurodegeneration (Liu et al. *Nature* 2012). However, not all phenotypes of mir-34 mutants were explained by Eip74A, and Eip74A, as an ecdysone-inducible factor, is not directly evolutionarily conserved in vertebrates (Wasylyk et al. *Eur J Biochem* 1993). A possible key functional target of mir-34 that is evolutionarily conserved is DH44-R2, a corticotropin releasing factor receptor (CRFR) (microRNA.org 2010).

Mammalian CRFRs are neuropeptide receptors whose ligands include corticotropin releasing factor (CRF) and urocortin 1, 2, and 3 (UCN1, UCN2, UCN3). This G-protein coupled receptor is a major regulator of the response to stress in both cells of the nervous system and in macrophages (Lowery and Thiele *CNS Neuro Disord Drug Targets* 2010, Tsatsanis et al. *FEBS* 2005). CRFR has been studied in the context of neurodegeneration through the mis-regulation of Tau-phosphorylation. Tau is a microtubule-associated protein; when hyperphosphorylated it forms aggregates that contribute to Alzheimer's disease progression (Rissman et al. *J Neuro* 2007). Additionally, CRFR was previously shown to activate the pro-apoptotic factors Bax and Bad (Tsatsanis et al. *FEBS* 2005). It is believed that the promotion of apoptosis could in part be through the activation of the ETS transcription factor pU.1, a close relative to Eip74A in *Drosophila melanogaster* (Tsatsanis et al. *J Immunol* 2006; Wasylyk et al. *Eur J Biochem* 1993). In mouse studies, it has been shown that mir-34 regulates CRFR in the brain, but CRFR's main mechanism of action in neurological disorders has not been determined (Haramati et al. *J Neuro* 2011). In *Drosophila*, the CRFR homologue DH44-R2 has not been studied in the context of neurodegeneration or miRNA regulation. **I propose that mir-34 prevents overexpression of DH44-R2 (CRFR) in the brain of *Drosophila melanogaster*. Over-expression of DH44-R2 results in an increase in Tau-phosphorylation and accompanying protein aggregates, contributing to**

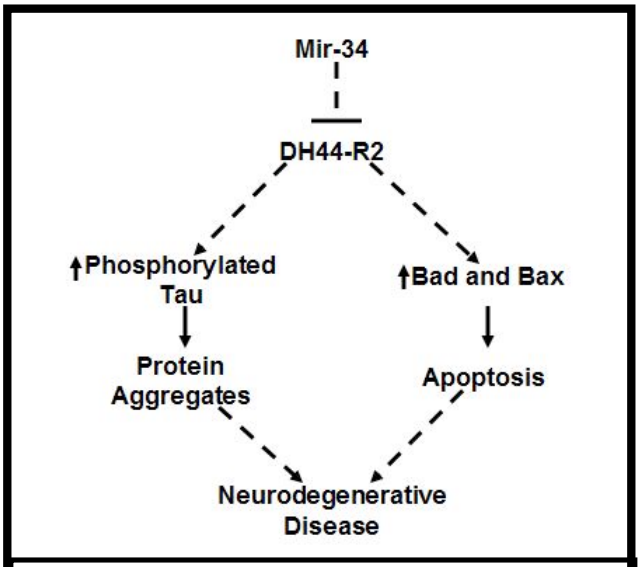

Figure 1: A model for DH44-R2 as a target of mir-34. Steps being tested in this proposal are indicated by dashed lines.

neurodegeneration. Additionally, DH44-R2 function could be activating apoptotic pathways to promote neuronal cell death (See Figure 1). The following aims are designed to test this model:

Aim1: Determine if DH44-R2 is a direct target of mir-34 and is up-regulated in the absence of mir-34 in the brain. DH44-R2 contains a possible conserved binding site for mir-34 in *Drosophila melanogaster*, but a functional interaction has not been shown. In this aim, I will test if DH44-R2 is bound and down-regulated by mir-34 *in vivo* in the brain. First, I will determine if protein levels of DH44-R2 are up in mir-34 mutants and whether this effect is mitigated by expression of mir-34. Next, I will use a luciferase transgene expressed in the brain containing the DH44-R2 3' UTR either with or without the putative mir-34 binding site and compare luciferase expression by western blot and fluorescence immunohistochemistry, either in the background of wild type or null mir-34. Simultaneously I will compare luciferase expression to DH44-R2 expression at the endogenous locus for control with the wild type DH44-R2 3' UTR.

Aim2: Analyze the effects of differential regulation of DH44-R2 on the progression of neurodegeneration under wild type or disease conditions. Mammalian CRFR is a major regulator in the response to stress in the brain; this role may be conserved in *Drosophila*. I will use a brain-specific transgene of DH44-R2 to up-regulate its expression as well as tissue-specific RNAi to DH44-R2 to specifically down-regulate its expression in the brain. Experiments will be performed in the presence or absence of the ataxin-3 polyQ transgene (Warrick et al. *Cell* 1998). Using brain histology and immunohistochemistry for stress-induced chaperones I will determine whether CRFR levels influence the formation of brain vacuoles and inclusion bodies or polyQ plaques in both genetic backgrounds. These measurements are readouts of the progression of neurodegeneration (Liu et al. *Nature* 2012).

Aim3: Determine whether DH44-R2's role in Tau phosphorylation and/or apoptotic signaling is necessary and/or sufficient to induce neurodegeneration in a disease background. In mammals CRFR signaling has been connected to the activation of the pro-apoptotic factors Bad and Bax and to an increase in the activity of Tau kinases and the downstream accumulation of phospho-Tau aggregates (Tsatsanis et al. *FEBS* 2005; Rissman et al. *J Neuro* 2007). To test the effect of DH44-R2 on Tau or the initiation of apoptosis, I will use as genetic backgrounds for all experiments either DH44-R2 transgenic over-expression or brain-specific RNAi in the presence or absence of the ataxin-3 polyQ transgene. I will perform western blot analysis comparing expression of apoptotic markers, activated Tau kinases, and levels of phosphorylated Tau. To check for differences in P-Tau aggregation I will separate soluble from insoluble protein fractions in the brain and visualize them via western blot or, alternatively, look for aggregates on blue native gel. To compare the number of apoptotic cells I will measure levels of phosphatidylserine on the outer membrane (Tsatsanis et al. *FEBS* 2005). I will also use a phospho-mimetic or non-phosphorylatable Tau mutant to determine if P-Tau is sufficient and/or necessary to initiate or suppress neurodegenerative phenotypes caused by DH44-R2. Finally, I will use over-expression and brain-specific RNAi for Bax and Bad to determine if activation of the apoptotic pathway is sufficient and/or necessary to initiate neurodegenerative phenotypes.

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